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RESEARCH ARTICLE

Fluidized-bed drying and storage stability of *Cryptococcus flavescentis* OH 182.9, a biocontrol agent of *Fusarium* head blight^{1,2}

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A method to produce dried granules of *Cryptococcus flavescentis* (formerly *Cryptococcus nodaensis*) OH 182.9 was developed and the granules evaluated for storage stability. Small spherical granules were produced and dried using a fluidized-bed dryer. A drying and survival curve was produced for the process of fluidized-bed drying at 30°C. The granules were dried to different moisture contents (4, 7, 9 and 12%) and evaluated for storage stability at 4°C for up to a year. These different moisture contents granules had the following respective water activities (0.22, 0.38, 0.47 and 0.57 a_w). The results show the storage stability varied significantly across this moisture content range. The 9% moisture content sample had the best short-term stability (up to 4 months), while 4% moisture content had the best long-term survival (1 year). A desorption isotherm of *C. flavescentis* was determined and modeled. The results of the storage stability and drying studies are interpreted in context of the desorption isotherm.

Keywords: *Cryptococcus flavescentis*; fluidized-bed; drying; desorption isotherm

Introduction

Cryptococcus flavescentis (formerly *Cryptococcus nodaensis*) OH 182.9 (NRRL Y-30216) is a basidiomycetous yeast that reduces *Fusarium* head blight (FHB), incited by *Gibberella zeae*, in greenhouse and field studies on wheat (Schisler, Khan, Boehm, and Slininger 2002; Khan, Boehm, Lipps, Schisler, and Slininger 2004). In an effort to facilitate the progression of *C. flavescentis* towards a commercially viable FHB biological control option, studies in our laboratory have been performed to optimize its production, bioefficacy (Zhang, Boehm, Schisler, and Slininger 2005a; Zhang et al. 2005b) and physiology (Dunlap, Evans, Theelen, Boekout, and Schisler 2006).

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¹Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

²Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Additional research is still needed to develop cost effective methods of drying and stabilizing *C. flavescentis* to produce a product with a suitable shelf-life. Optimization of the formulation and application parameters are critical steps in the biocontrol product development process.

Wettable powders and granules have long been a common method of formulating microbial biological control agents (Burgess and Jones 1998). Successful wettable powders and granules offer ease of use, convenience for transportation, long shelf-life and consumer acceptance (Burgess and Jones 1998). These dried products can be formed by a variety of methods such as, air drying, spray drying or fluidized-bed drying. Spray drying and fluidized-bed drying have traditionally been used to produce active dry yeast for the food industry due to their reliability, low costs and high throughput (Grabowski, Mujumdar, Ramaswamy, and Strumillo 1997; Luna-Solano, Salgado-Cervantes, Ramirez-Lepe, Garcia-Alvarado, and Rodriguez-Jimenes 2003; Yuzgec, Turker, and Becerikli 2004; Luna-Solano, Salgado-Cervantes, Rodríguez-Jimenes, and García-Alvarado 2005). Fluidized-bed drying is generally considered the less stressful of the two for drying microbial cells, since it involves less extreme water loss and temperature gradients (Larena, Melgarejo, and Cal 2003; Morgan, Herman, White, and Vesey 2006). Fluidized-bed drying is typically a method used to dry granulated solids. For example, Baker's yeast has been harvested as a filter cake and extruded into pellets for drying. Alternatively, products can be introduced into a fluidized-bed dryer as liquid by spraying it on to a granulated inert material. This can be used to spray coat a granule with cells or used to form a spray agglomeration, where the spray is used to bind small solid particulates together to form a larger granule.

The objective of the current study was to evaluate the potential of fluidized-bed drying to produce viable dried preparations of *C. flavescentis*. In the course of our investigation we developed a method of producing moist granules of *C. flavescentis*, which could be easily introduced into the fluidized-bed dryer. In addition, we determined the storage stability of these dried granules under different moisture content/water activities for up to a year. We measured the desorption isotherm of the yeast cells to better understand the thermodynamics of the drying process.

Materials and methods

Biomass production

Cryptococcus flavescentis OH 182.9 (NRRL Y-30216) was produced in a B Braun D-100 fermentor charged with 80 L of SDCL medium (Slininger, Schisler, and Bothast 1994). Antifoam 204 (Sigma, St Louis, MO) was added prior to medium sterilization and cultures were not pH controlled after inoculation at pH 7.0. To initiate a production run, log-growth cells of OH 182.9 served as a 5% seed inoculum. The fermentor was operated at 25° C, 20 L/min aeration and 200 rpm agitation provided by twin rushton impellers. Eighteen hours after inoculation, the temperature was reduced to 15° C to cold shock the cells for 24 h prior to harvest. After completion of biomass production at approximately 42 h, colonized reactor broth was concentrated into a paste using a Sharples 12-V tubular bowl centrifuge. The cell paste was frozen at -80° C until use.

Granulation

Uniform spheres containing cells of *C. flavescentis* were produced by dropping droplets of 10% (w/w) aqueous suspension of the cell pellet into a rotating bed of perlite (Harborlite 1500 S, Harborlite Corp. Santa Barbara, CA) using a 20-gauge needle and peristaltic pump. The rotating bed was a modified seed coater spinning at approximately 45 rpm. Excess free perlite was removed with sieving with 1-mm screen.

Fluidized-bed drying

Fluidized-bed drying was performed with a Niro-Aeromatic fluid bed dryer type STR-1 (Niro-Aeromatic Inc., Columbia, MD). Five hundred gram batches of the wet *C. flavescentis* spheres were dried at 30°C and an air volume of 90 m³/h. Five replicate dryings were performed. The relative humidity of the ambient air was ~50% with no humidity controls in place. Samples were taken every 3 min of drying and assayed for moisture content and viability. Moisture content ((wet-dry)/wet) of the samples was determined with a moisture analyzer (Mark I, Denver Instruments, Tempe, AZ).

Viability testing

Air-dried samples were resuspended in 50 mL of weak (0.03%) phosphate buffer at 25°C, mixed in a Stomacher 80 (Seward Laboratory Systems Inc, Bohemia, NY) with the normal setting for 60 s. Serial dilutions were made for each sample and plated on TSBA/5 media. Plates were incubated at 25°C for 2 days until colony counting.

Desorption isotherm

Desorption isotherm was determined using the standard saturated salt solution method (Labuza 1984). The cell paste recovered from the production media was spread uniformly across a weigh boat (~0.5 g) and maintained over saturated salt slurries at 25°C. The samples were weighed daily until the mass variation for a sample was less than 1 mg. The data was fit to Brunauer–Emmet–Teller (BET) (Brunauer, Emmett, and Teller 1938) and Guggenheim–Anderson–de Boer (GAB) models (Schär and Rüegg 1985) using Sigmaplot 9.0 (Systat software Inc., San Jose, CA).

Results and discussion

Fluidized-bed drying requires the product to be in granular form for the most cost-effective method of drying. The simplest method of forming a granule is to extrude a paste or filter cake of cells into small pellets. However, preliminary studies demonstrated that *C. flavescentis* cannot be readily formed into filter cakes. When *C. flavescentis* is filtered, it rapidly forms a water impenetrable layer that terminates further filtering. Figure 1A is a scanning electron micrograph showing the cross-sectional area of a membrane clogged by *C. flavescentis*. The figure shows

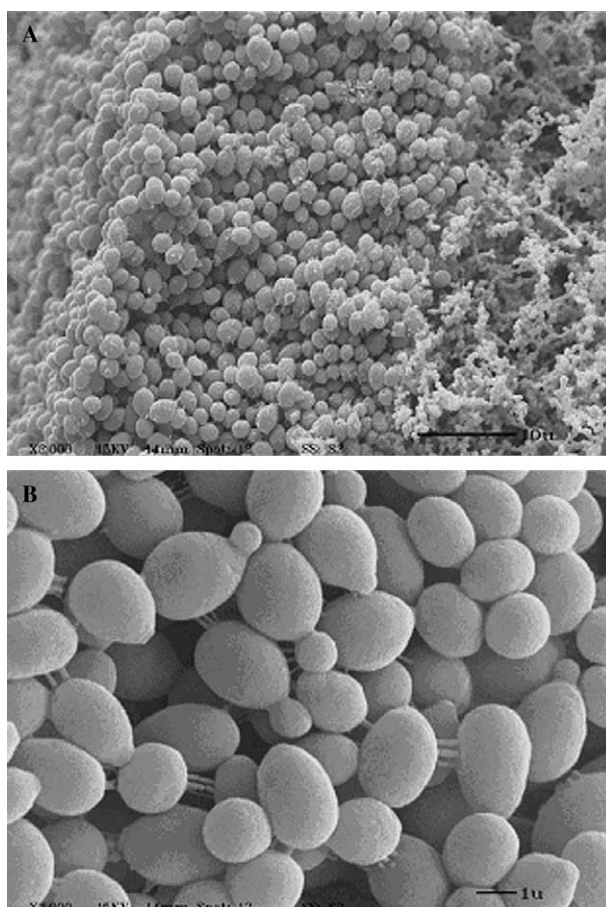


Figure 1. Scanning electron micrograph of (A) a *C. flavesceus* fouled filter membrane and (B) *C. flavesceus* with a secreted exudate.

C. flavesceus is capable of clogging the membrane with a $\sim 25\text{-}\mu\text{m}$ layer of cells. Figure 1B shows a close up of the cells and it appears they secrete an exudate which may contribute to the clogging. *C. flavesceus* does not flocculate in solution under the current growth conditions. This not only limits the options which can be used to produce granules, it also limits how the cells can be harvested and the initial dewatering step. Harvesting on a pilot plant or larger scale is now limited to methods that rely on centrifugation as a means of concentrating the cells. This typically results in a slurry of cells rather than a dense pellet, which must be formed into a granule. One solution would be to add an inert which would produce a paste and granulated through extrusion. This is less than ideal, since it limits the percentage of active ingredient in the resultant dried product and would greatly increase the amount of material needed to treat an area. An alternative approach could be to explore nutritional parameters of the medium used to produce *C. flavesceus* to identify conditions that may produce biomass less prone to filter clogging.

Because the current production protocol and medium composition produces highly effective, stress tolerant cells (Dunlap et al. 2006), a simple process for

forming small (~ 3 mm) spheres from a slurry of *C. flavescentis* cells was developed for the current study in order to provide an uniform product for fluidized-bed drying trials. Spheres were produced by dropping droplets of the cell slurry into a rotating inert powder (perlite). This allowed us to produce non-adherent, soft granules with a minimum of inert material. The granules had good flow behavior in the dryer with no problems of clumping and easily formed a fluidized-bed.

The drying profile of the granules was determined in a batch fluidized bed dryer at 30°C. The drying was limited to this temperature since lower temperatures are generally not as deleterious to cell viability. In addition, it is easier to harvest discrete fractions at different moisture contents under these slower drying conditions. Under these conditions, the granules dried in less than 30 min and provided the opportunity to sample the granules at different moisture contents during the drying course. The results of fluidized bed drying of *C. flavescentis* are presented in Figure 2. Moisture content and viability of the cells were monitored over the course of drying. The results show *C. flavescentis* viability was well maintained until the moisture content decreased below 20%. Below 20% moisture content, a gradual loss of viability is observed until a final moisture content of 2–3%. The viability loss across this range of moisture is on the magnitude of one log.

Figure 3 shows the rate of water loss at different times during the drying curve. Fluidized bed drying curves can be characterized by three phases with different drying rates. The increasing-rate phase is the initial period of drying, when the granules are warming to the equilibrium temperature of the dryer and the drying rate is increasing. The next period is the constant-rate region, characterized by the constant level of moisture loss. The final drying period is the falling-rate region, in which water loss is limited by the rate of water transport within the granule. This can be from the water needing to diffuse from the interior of the granule to the surface or removing water that is more tightly bound to the granule. The data shows cell viability begins to suffer in conjunction with the falling rate period, approximately at the 15-min mark of the drying curve. Then cell viability continues to decrease as additional water is removed from the granule. Previous studies with *S. cerevisiae* show a similar phenomenon (Beker and Rapoport 1987).

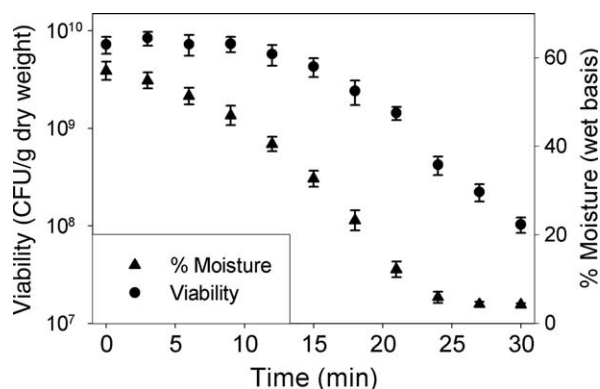


Figure 2. Drying curve and viability of *C. flavescentis* during fluidized bed drying at 30°C. Error bars are reported at ± 1 SE.

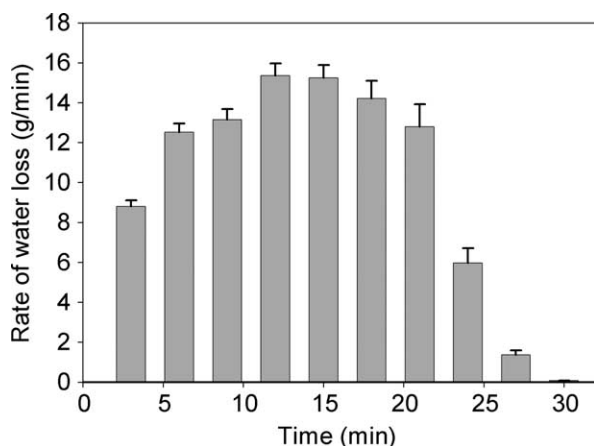


Figure 3. Rate of water loss for granules of *C. flavescentis* during fluidized bed drying at 30°C. Error bars are reported at ± 1 SE.

To better understand the role of water–yeast interactions, a desorption isotherm for *C. flavescentis* at room temperature was determined. The desorption isotherm of *C. flavescentis* is shown in Figure 4. Sorption/desorption isotherms describe the relationship of moisture content to water activity for a material at equilibrium and at a specified temperature. The results show a typical sigmoid isotherm, which is common for biological materials. Similar isotherms have been reported for *S. cerevisiae* (Koga, Echigo, and Nunomura 1966) and a *Lactobacillus* strain (Linders, de Jong, Meerdink, and van't Riet 1997). Desorption isotherm data are typically analyzed using mathematical models. Two common models used for analysis are the BET (Brunauer et al. 1938) and GAB models (Schär and Rüeegg 1985). The BET model is given by:

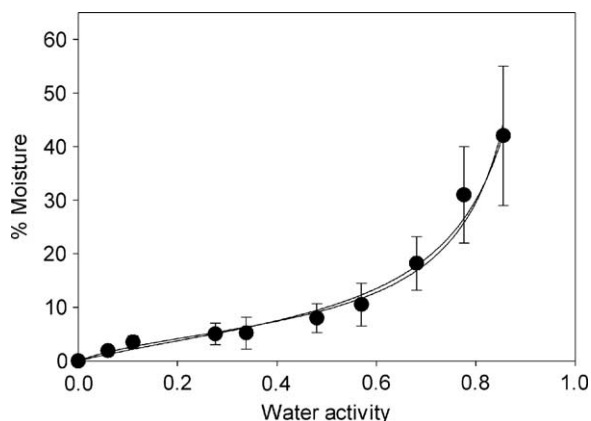


Figure 4. Desorption isotherm of *C. flavescentis* at 25°C. Error bars are reported at ± 1 SD. R^2 for the GAB and BET equations are 0.98 and 0.97, respectively. The fitted parameters for the GAB and BET equations are found in Table 1.

$$X = X_{\text{BET}} \frac{Ca_w}{(1 - a_w)(1 + (C - 1)a_w)}$$

In the BET model, X is moisture content, X_{BET} is the BET monolayer moisture content, a_w is water activity, C is a constant related to the net heat of sorption. The GAB model is given by:

$$X = X_{\text{GAB}} \frac{C'Ka_w}{(1 - Ka_w)(1 - Ka_w + C'Ka_w)}.$$

In the GAB model, X is moisture content, X_{GAB} is the GAB monolayer moisture content, a_w is water activity, C' and K are constants related to the temperature effect.

Both models were fit and plotted to the experimental data in Figure 4. The calculated value of monolayer moisture content and the constants are summarized in Table 1. Both models provided similar monolayer moisture content determinations. Monolayer moisture content is a concept that describes a condition where a single layer of water molecules covers the surface of the material. The water in this layer is considered tightly bound, and is associated with low water activities in the isotherm. Subsequent water layers are less tightly bound and represent the plateau region in a sigmoid isotherm. The highest layers of associated water are only weakly associated and represent the high water activities on the right side of the isotherm. A past study with *S. cerevisiae* (Koga et al. 1966) showed the weakly associated water at high moisture contents had a heat of vaporization almost equal to that of pure water (~ 10 kcal/mol). Then the heat of vaporization gradually increases with decreasing moisture content, until reaching ~ 20 kcal/mol at 5% moisture in yeast. This quantifies the strong interaction and binding of water to the cell surface at low water activities.

The next step in our investigation was to determine the storage stability of the granules. The storage stability of the fluidized bed dried granules were evaluated for refrigerated storage (4°C). In addition, preparations dried to different moisture contents were examined to identify the optimum moisture content for storage. The results for four moisture contents (4, 7, 9 and 12% (wet basis)) are summarized in Figure 5. The four preparations differed considerably in their storage stability. The 12% moisture content sample had the worst shelf-life losing more than a Log per month for the first three months. The 9% preparation demonstrated good short-term stability losing only a half a Log during the first 4 months. Interestingly, the preparation experienced a rapid drop in viability after four months of relatively stable survival. The 4% preparation had the best long-term shelf-life, losing approximately 1.5 Log after one year in storage. These results suggest the optimum moisture content for storage is dependent on the anticipated storage time. For

Table 1. Derived desorption isotherm model parameters.

Model	χ	C	K
BET	6.5 ± 0.5	3.5 ± 1.3	
GAB	5.4 ± 0.8	6.1 ± 3.4	1.03 ± 0.03

Based on equilibrium measurements at 25°C , error reported at ± 1 SD.

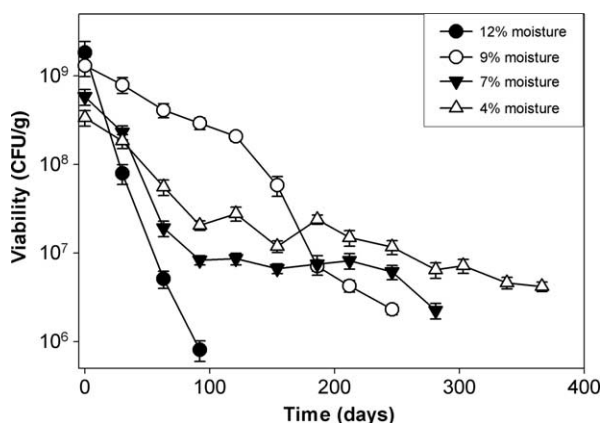


Figure 5. Storage stability of granules of *C. flavescentis* at 4°C and different moisture contents. Error bars are reported at ± 1 SE.

comparison, the optimum moisture content reported for storage of baker's yeast is 7.0–8.5% with no specified time range indicated (Beker and Rapoport 1987).

Desorption isotherms are particularly helpful in understanding drying phenomena and in selecting storage moisture content conditions. Interpreting the storage stability results in context of the desorption isotherm provides a rational basis for guiding storage condition decisions for similar organisms. The optimum moisture content for storage for most reported organisms lies in the middle of the plateau region of the desorption isotherm. This region of the desorption isotherm for microorganisms is represented by water activities in the 0.2–0.6 range. This is below the water activity that can support microbial growth (~ 0.7) and above the typical monolayer moisture content (~ 0.20). Figure 6 shows the placement of the storage conditions on the desorption isotherm. All the points lie on the middle plateau of the sigmoid curve. Granules containing OH 182.9 that were stored at 12% moisture ($\sim 0.57 a_w$) consistently lost CFUs over time while those containing 9% moisture ($\sim 0.47 a_w$) were stable for 4 months (Figure 5). It is probable that cells stored under

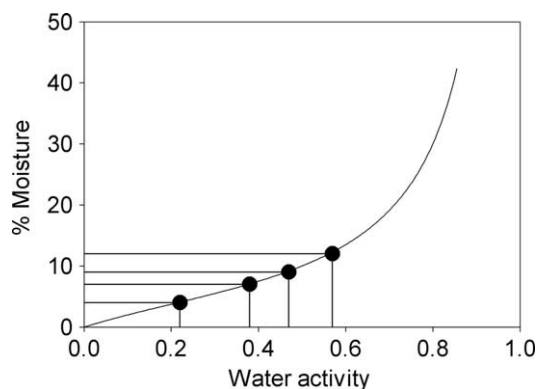


Figure 6. Overlay of storage stability conditions on the desorption isotherm of *C. flavescentis*.

the 2 moisture regimes differed considerably in their underlying metabolic activity which would account at least in part to the storage stability differences observed. It is important to note that the desorption isotherm was determined at 25°C and the storage stability was preformed at 4°C. In general, the entire desorption isotherm curve would be shifted slightly upward with lower temperatures and downward with higher temperatures. The shape of the curves remains similar and would not differ significantly for the purpose of this discussion. It is recommended that this area of the desorption isotherm be given first consideration when optimizing storage stability conditions.

Our results demonstrate the importance of being able to control the drying kinetics during water removal so that a desired moisture content of the final product can be achieved. The storage stability experiments highlight the importance of this observation. The ability to know or predict when the desired moisture content has been achieved is an important parameter when developing drying procedures. It is often not practical to stop and determine moisture content on a pilot plant or commercial scale. Our results demonstrate the importance of process control in being able to reproducibly manufacture commercial quantities of a high CFU, dried OH 182.9 product with a consistent shelf life.

In summary, the current study identified a simple method of producing granules from a microbial slurry. The method is applicable to other organisms, scalable and allows for easy incorporation of a number of adjuvants. The work demonstrates fluidized-bed drying is a viable option for the drying of granules of *C. flavescentis*. The storage stability of different preparations with varying moisture contents were determined for up to a year at 4°C. The results identified significant changes in storage stability with moisture content. A desorption isotherm for *C. flavescentis* was reported and used to interpret the storage stability results. The results suggests guidelines for identifying the optimum moisture content for storage based on their desorption isotherm.

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